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THIRD ANNUAL REPORT

February 1, 1991 - January 31, 1992

"Evaluation of Dried Storage of Platelets and RBC for Transfusion: Lyophilization and other Dehydration Techniques"

Grant No. N00014-89-J-1712
From the Office of Naval Research:
Navy Medical Research and Development Command
Department of the Navy

Performance Sites and Investigators:

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American Red Cross Blood Services-Mid Atlantic Region:
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Respectfully submitted, April 21, 1992.

INTRODUCTION

Project description: To explore and develop protocols for drying blood platelet preparations for extended storage without undue compromise of hemostatic function or cell integrity upon rehydration. A successful project outcome would greatly facilitate the use of platelets in the medical support of combat casualties with severe blood loss, major surgical repair, or extensive burn injury. The three collaborating performance sites are bringing together expertise in cell stabilization techniques and platelet function testing both in vitro and in vivo. The ultimate goal is to identify a practical procedure to produce a stabilized platelet preparation that can be stored virtually indefinitely while maintaining transfusion efficacy. Such a product would also be useful in civilian blood banking.

The third year of this grant saw advancement of the animal model studies testing the hemostatic capabilities and circulatory survival of dried platelets stabilized with paraformaldehyde, refinement of an alternative platelet stabilization method using permanganate and trehalose, and an initial study of dried RBC in an in vivo circulatory model. In normal canine experimental subjects, rehydrated canine platelets were found in circulation for at least 24 hours after infusion; similar results were obtained in bleeder pigs (a congenital strain with von Willebrand's disease). Rehydrated platelets were shown to adhere to subendothelium in situ when the animal subjects were exposed to a vessel stenosis and injury protocol or subjected to a bleeding time test. Testing was also performed, although not previously planned, with a new instrument under development to assess an in vitro bleeding time for platelet or whole blood preparations. Our results show that rehydrated human platelets can substitute adequately for fresh platelets. Further such testing will be limited by the availability of this instrument.

Progress on development of the permanganate stabilization and freeze-drying protocol for platelets was hampered by six months of downtime on the shelf lyophilizer unit. However, we processed and evaluated ten separate human blood platelet preparations with subdivided protocols to optimize each step within the basic procedure. Arrangements are now being pursued to obtain large quantities of canine or swine blood for permanganate preparations suitable for use in the animal models already being employed in testing of the paraformaldehyde-stabilized platelets. We plan also to repeat testing of dried RBC in animal models to extend our findings in the initial successful circulation of rehydrated canine RBC in a normal dog.

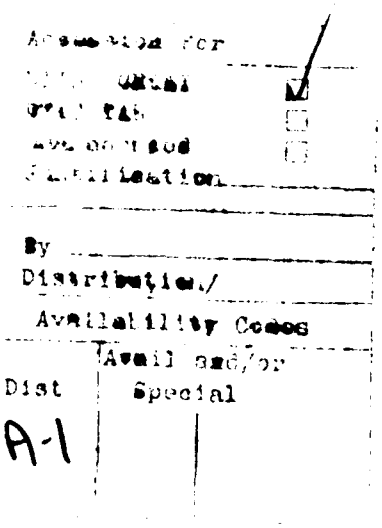
PATENTS AND EXTENSION OF PROJECT

No further publication of abstracts since noted in the last annual report, nor any submission of manuscripts has occurred related to this project activity because of declaration of interests in pursuing patent protection of the subject invention. Disclosures were made by the investigators to the institutional patent committees in June, 1991, followed by generation of inter-institutional patent sharing agreements and notification of the Office of Naval Research of election of title by the Universities. The biomedical research division of Armour Pharmaceutical Co., has agreed to help support the filing of a patent and to further commercialization of the freeze-dried platelet product. However, as

of the date of this writing, no filing has yet been made in the U.S. Patent Office.

A no-cost extension of this project was granted thru July 31, 1992, to support work originally defined in the project workscope but delayed by breakdown of the lyophilizer. In addition, another three year workplan has been approved and funded by the Office of Naval Research to carry this project further toward preclinical human trials of freeze-dried platelets. The objectives in the new grant include safety and toxicity testing of these preparations, extensive evaluation of physiologic and functional properties of rehydrated platelets, and the practicality of making sterile preparations in unit dose proportions. One spin-off advantage of the paraformaldehyde and permanganate protocols already under development is that those stabilization agents may be effective antimicrobial and antiviral methods in reducing the risk of transmission of infectious pathogens by transfusion products. The blood supply may actually be made much safer in terms of infectious risk with these preparation protocols.

Attached are subcontract reports from the other performance sites. Specific details on process procedures, especially refinement of paraformaldehyde or permanganate stabilization steps and addition of water replacement molecules must be kept from publication until a patent application has been filed. This should occur before the final report is due at the end of the no-cost extension.



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Third Annual Report

February 1, 1991-January 31, 1992
(Grant extension, without additional funds, to July 31,1992).

Dehydration of Platelets and RBC: Long Term Storage of Transfusion Products

UNC/ECU Contract: Grant No. N00014-89-J-1712 from the Office of Naval Medical Research and Development.

Performa- site: University of North Carolina at Chapel Hill.

Principal Investigator: Marjorie S. Read, Ph.D.

Co-PI: Robert Reddick, M.D.

Submitted: April 8, 1992.

The purpose of this study was to prepare dried/rehydrated platelets and RBCs that could be used as transfusion products. We proposed to use rehydrated platelets as substitutes for or addition to fresh platelets to restore hemostasis without pathologic thrombosis.

A. Studies conducted during 1991.

During the past year we have produced a rehydrated platelet product that has been safely infused into animals, normal pigs and dogs and bleeder pigs. We have collected blood samples at time intervals and established that rehydrated platelets will circulate for at least 24 hours. The rehydrated platelets were found to adhere to the bleeding time wound edge and to injured arteries. Rehydrated platelets were also found in formed thrombi and in some organs.

1. Refinement of the dried platelet procedure. During the year adjustments and changes have been made in time of fixation, and in the temperature and concentration of fixative. These parameters are important and critical to the preservation of the surface receptors necessary for normal agglutination and adhesion of platelets.

2. Chemical labeling of dried and rehydrated platelets. Since we are using rehydrated platelets in live animals which must be subjected to surgical procedures, we do not wish to use radioisotopes to label blood cells. Instead, we have selected a fluorescent label which labels the lipid in the cell membrane and does not appear to leak or leach from the cell. Conditions for successfully labeling blood cells must be determined by trial and error. All protein must be removed from the mixture in order for the cells to take up the dye. An overabundance of dye will rupture the cells. We have successfully labeled both platelets and RBCs and have found appropriately labeled cells to be safe for in vivo studies. The ability to label cells with markers other than radioisotopes has greatly enhanced our ability to work with these cells in animals.

3. Studies of hemostatic effectiveness of rehydrated platelets infused into normal pigs and dogs. Rehydrated platelets were found to remain in circulation for at least 24 hours in a normal dog. When animals were subjected to the stenosis and injury protocol, it was found that rehydrated, labeled platelets were present in the microthrombi and adhering to the denuded subendothelium. Labeled platelets were also seen along the wound edges and in organs such as the spleen, liver, and lung. The presence of these labeled rehydrated platelets in organs indicated that these cells did circulate, and were not immediatly

removed. Platelets seen in the clotted blood were adherent to fibrin strands along with fresh platelets.

4. Studies with rehydrated platelets in bleeder pigs. Rehydrated platelets were infused into a bleeder pig which also underwent the stenosis and injury protocol. Results were mixed. Few platelets were seen to adhere to the bleeding time wound, an expected result, since bleeder pigs have no von Willebrand factor. However, they did circulate and were present in areas of the lung that had trauma injury. This finding could not be fully analysed since uninjured sections of the lung were not available for comparison. However it was promising that rehydrated platelets were in the injured areas. In this animal, rehydrated platelets alone did not correct the bleeding time, which was not totally unexpected. The volume of platelets given was probably not sufficient to correct bleeding time even if vWF had been added. In other studies with two bleeder pigs with trauma injuries to the tail, the pigs were treated with rehydrated platelets. Bleeding was temporarily arrested, but recurred when the injured tail was brushed against the cage or housing wall. There were no indications of any adverse reactions to the infused rehydrated platelets even though the preparation was from several different pigs. Further in vivo studies are needed to confirm and document the hemostatic value of the rehydrated platelets.

5. Development of an in vitro bleeding time test for evaluation of rehydrated platelets prior to in vivo testing. We have begun testing each batch of rehydrated platelets with an instrument called the Clot Signature Analyzer (CSA) prior to infusion into animals. This instrument is in the clinical testing stage as an in vitro means of determining bleeding time (BT). Anticoagulated blood samples are passed through tubing under controlled conditions. A small hole is punched in the tubing wall. As blood passes through the opening, platelets are deposited on the edge of the punched tubing. The time to build up of platelets to close the opening and prevent "bleeding" is taken as the in vitro bleeding time. We have tested normal blood and normal blood with normal platelets replaced with rehydrated platelets. We have also tested von Willebrand deficient whole blood and vWD blood with vWD platelets replaced with rehydrated normal platelets. Results to date indicate that rehydrated platelets can be successfully substituted for fresh platelets. Rehydrated platelets produced a bleeding time comparable to that of fresh platelets in the human system. Additional work needs to be done to evaluate canine rehydrated platelets which are being used in the animal model. The use of this instrument offers us a means to evaluate individual platelet batches prior to whole animal experiments. We have data on both pigs and dogs, but completion of the testing is being held up due to repairs to

the instrument which had to be shipped back to the manufacturer. The CSA is one of the most promising new in vitro BT test systems presently avaible.

6. Studies with rehydrated RBC. We have continued to develop methods of preserving red blood cells that will allow us to dry and rehydrate the cells. To date we have several protocols for red cell preservation. Only one preparation has been infused into a dog. The rehydrated RBC were labeled with a fluorescent dye and blood samples collected for study. Rehydrated RBC were observed in circulation with no evidence of adverse reaction. The dog was sacrificed and tissue samples taken for study.

B. Site visit held at UNC for ONR Grant renewal.

We were site visited in Chapel Hill, N. C. on November 21, 1991. Site visitors were Captain Stephen Lewis and Dr. Robert Voleri. Participants were Dean Bondurant, Dean, UNC School of Medicine, Dr. Grisham, Chairman, Department of Pathology, Dr. Jacobs, ECU Vice Chancellor, Dr. Roberts, The Director of Hemostasis and Thrombosis at UNC, and the principle investigators, Drs. Read, Reddick and Bode.

C. Patent. We have been negotiating with Armour to produce a patent application for the rehydrated platelets as therapeutic products. The patent application is now in the process of being drafted.

D. Publications. Publications are being withheld until the patent application is filed. We anticipate at least two publications immediately post filing.

PROGRESS REPORT ON LYOPHILIZED SAMPLES 91/92 FROM ARC, MID ATLANTIC REGION

LYOPHILIZED PLATELET SAMPLES

We have tested four lyophilized platelet samples processed with 0.01 % KMO4 + 500mM Trehalose/Tris. The samples were washed and resuspended in Unisol platelet storage medium for in vitro testing.

2 mL of Unisol was added to the samples with resuspension of the lyophilized platelets. Platelet count and size distribution were performed using a Coulter counter to measure platelet yield and mean volume.

Platelet shape change with ADP and the hypotonic shock response were tested in a Chrono-log Aggregometer and responses determined by changes in light transmission.

The average platelet counts of the samples were 1.06 x 10^6 with a normal size distribution and a mean volume of 7.2 um^3 which is normal and does not indicate swollen or fragmented platelets. This was confirmed by morphologic examination of the sample which showed on the average 46 % with normal discoid morphology. However, the platelet did not respond to extent of shape change with ADP or had any recovery response to hypotonic shock, indicating that they were not functional.

LYOPHILIZED RBC SAMPLES.

Rehydration of eigth lyophilized red cell samples using either KMnO4, trehalose or sucrose as preservatives were performed by addition of red cell preservation medium used in storage of red cells. As shown in attachement the rehydrated samples contained less than five percent intact red cells. Part of the problem could be the resuspension medium . We plan to investigate the suitability of various commercially available RBC additive solutions used in storage as resuspension medium. Once we have been able to obtain non-hemolyzed lyophilized RBC, testing will be performed to measure deformability by filtration and viability by levels of ATP.

PROPOSAL FOR STUDIES THE COMING YEAR

INVESTIGATION OF PHYSIOLOGIC, METABOLIC AND FUNCTIONAL PROPERTIES OF LYOPHILIZED PLATELETS.

Background and objective.

A very promising finding last year was that ligthly paraformaldehyde fixed platelets tolerated lyophilization, and after resuspension in citrated plasma demonstrated well preserved morphology and maintenance of functional properties such as adhesion and a shape change response to the agonist ADP. This suggested that these platelets were still viable with intact metabolic and functional properties.
The main goal in the the forthcoming year will be to establish the maximal exposure in time and concentration to paraformaldehyde of the platelets that is needed for lyophilzation, while still maintaining normal platelet physiologic, metabolic and functional properties.

Protocol.

1 day old standard CPD-anticoagulated platelet concentrates (PC) will be obtained from ARC, Mid-Atlantic Region.
Samples of 5 mL PC will be exposed to different concentations of paraformaldehyde (0.25 % - 2 %) in a phoshate/acetate containing platelet storage medium (Setosol) for various periods of time (10-60 min) at 20-24 C. A volume ratio of 1 part PC to 3 parts paraformaldehyde-Setosol will be used.

After the fixation samples will be counted and be sent to Dr. M. Read, Chapel Hill for lyophilization. The lyophilized samples will be returned to the ARC laboratory in Norfolk for evaluation of platelet yield and metabolic and functional properties.

In vitro assays.

The Setosol suspended platelets will be washed once and resuspended in autologous CPD-plasma for in vitro testing. Comparison will be performed with stored standard CPD-anticoagulated platelet concentrates.

Platelet spreading on glass.
A small sample will be added to a microscopic slide to examine the ability of the platelets to spread on glass. This property involves the change of platelet morphology from smooth discs to a pancake appearance with pseudopods and requires the intactness of the platelet cytoskeleton and normal energy metabolism.

Platelet respiratory rate, glycolytic rate and levels of adenine nucleotides.

These measurements of platelet energy metabolism will be performed as described previously. Briefly, CPD-plasma diluted PC (count: 300,000 plts/uL) will be transferred to an air-tight syringe and incubated for 2 hours at 37 C in a water bath. Before and after incubation samples will be taken for measurement of oxygen uptake, glucose consumption, lactate production and levels of ATP, ADP AMP and hypoxanthine. pH, pO2, pCO2 levels will be determined with a gas analyzer, levels of lactate and glucose by standard enzymatic assays, adenine nuleotides and their breakdown product hypoxanthine by HPLC.

ATTACHMENT

Red blood cells

All of the samples were thawed out or 1 ml. of the storage medium Unisol was added to the lyophilized samples for resuspension. The samples were examined under the microscope. The majority of the cells in all the samples were ghost cells with few remaining intact red blood cells.

1. Sample 901022 1M Trehalose 25mM KCl ETOH
   Less than 1% intact red blood cells (rbcs).

2. Sample 901022 800mM Trehalose 25mM KCl ETOH
   5% intact rbcs.

3. Sample 901016 250mM Sucrose 25mM KCl ETOH
   Less than 1% intact rbcs.

4. Sample 901016 250mM Trehalose 25mM KCl ETOH
   Less than 1% intact rbcs.

5. Sample 901022 800mM Sucrose 25mM KCl ETOH
   Less than 1% intact rbcs.

6. Sample 901022 1M Sucrose 25mM KCl ETOH
   1 to 2% intact rbcs.

7. Sample 910201 0.5% $KMnO_4$ 1M Sucrose
   5% intact rbcs.

8. Sample 910201 1.0% $KMnO_4$ 1M Sucrose
   Less than 1% intact rbcs.